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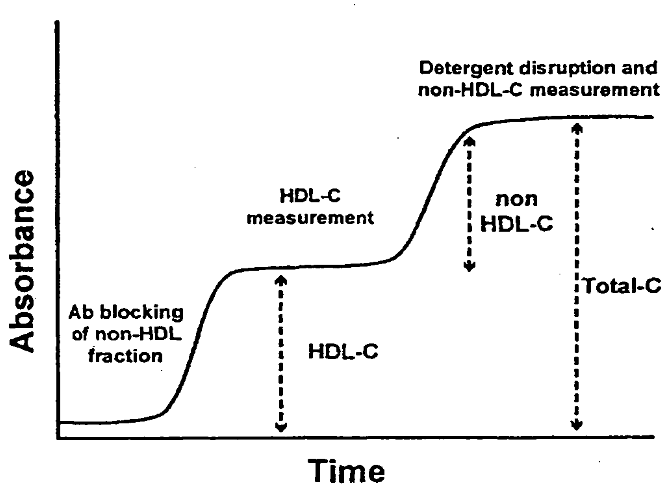
(54) Title: HOMOGENEOUS TESTS FOR SEQUENTIALLY DETERMINING LIPOPROTEIN FRACTIONS



(57) Abstract: The invention provides new homogeneous assays for the determination of the amount of LDL-C, of HDL-C, and of total cholesterol present in a sample. The method comprises complexing a first lipoprotein fraction with a complex-forming agent, such as an antibody, using enzymes to detect cholesterol in the non-complexed lipoprotein fraction, measuring the amount of cholesterol in the non-complexed fraction to provide a first cholesterol value, and then dissociating the complexed lipoprotein fraction from the complex-forming agent so that that cholesterol is available to be a substrate for the enzymes. The total amount of cholesterol present in the sample can then be determined. Further, the first cholesterol value obtained can be subtracted from the total cholesterol to obtain a value for the first lipoprotein fraction present in the sample. Optionally, a triglyceride assay can then also be performed on the sample in the same tube.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

HOMOGENEOUS TESTS FOR SEQUENTIALLY DETERMINING LIPOPROTEIN FRACTIONS

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. provisional patent application 60/136,709, filed May 28, 1999, the contents of which are hereby incorporated by reference.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention is owned by the United States Government.

BACKGROUND OF THE INVENTION

Serum total cholesterol (total-C) is an important risk factor for coronary artery disease (Wilson, P. *et al., Circulation* 97:1837-47 (1998)). The measurement of the cholesterol content of the lipoprotein fractions, however, is more valuable in establishing the risk for coronary artery disease, because the various lipoprotein fractions do not have the same effect on the process of atherosclerosis. Low density lipoprotein ("LDL," cholesterol associated with LDL is known as low density lipoprotein-cholesterol, or "LDL-C") is a pro-atherogenic lipoprotein fraction and more closely correlates with the risk for coronary artery disease than does total-C. In contrast, high density lipoprotein ("HDL," cholesterol associated with HDL is known as high density lipoprotein-cholesterol, or "HDL-C") is a negative risk factor for atherosclerosis (Gordon T, *et al., Am J Med* 62:707-14. (1977)). HDL is believed to be beneficial in reversing the process of atherosclerosis because of its ability to increase reverse cholesterol transport, a pathway by which excess cholesterol is transported from peripheral cells to the liver for excretion (Badimon J.J. *et al., Circulation* 86:86-94 (1992)). Recent recommendations for screening of cholesterol as a risk factor for coronary artery disease include the measurement of both LDL-C and HDL-C (The Expert Panel. *JAMA* 269:3015-23 (1993)).

Until recently, the most common procedure for determining LDL-C and HDL-C involved performing three tests, namely a measurement of total-C, a measurement of total triglyceride, and a measurement of HDL-C. HDL-C has often been determined after the precipitation and the physical removal of the apoB-containing

lipoproteins (the apoB containing lipoproteins are considered to be chylomicrons, LDL, and very low density lipoproteins, or "VLDL") by centrifugation. For example, Polymedco (Cortland Manor, NY), provides a test based on magnetic bead precipitation method. LDL-C can be calculated from the total-C, HDL-C and triglyceride values by the Friedewald equation (Friedewald W.T. *et al., Clin Chem* 18:499-502 (1972)). The above procedure, however, has several limitations (Schectman G., *et al., Clin Chem* 42:732-737 (1996)) and is relatively costly when used as a screening test because multiple tests are required and because of the complexity of the tests, in particular of the HDL-C determination.

Various additional approaches to HDL measurement have been developed, such as those described in Kerscher, U.S. Patent No. 4,892,815 ("Kerscher I"), and Kerscher, U.S. Patent No. 4,851,335 ("Kerscher II"). One recent improvement in lipoprotein cholesterol fraction measurement is the use of homogenous assays for HDL-C, that is, assays which do not require the physical separation of HDL or of the apoB-containing lipoproteins to make the measurements. Since homogenous HDL-C assays can be performed in one tube, they are easier to perform, easier to automate, less costly, and offer superior analytic performance (Lin M. *et al., Clin Chem* 44:1050-52 (1998); Rifai N. *et al., Clin Chem* 44:1452-58 (1998); Nauck M. *et al., Clin Chem* 44:144-51 (1998)). *See also*, Ziegenhorn, U.S. Patent No. 4,544,630, and Miki, European Patent Application EP 0 754 948 A1.

One of the approaches taken to determining HDL-C homogeneously has been to add an anti-apoB antibody to serum to form an antibody-antigen complex, after which HDL-C is measured. For example, Sigma Diagnostics (St. Louis, MO) markets the EZ-HDL™ cholesterol reagent kit. This kit permits measurement of HDL-C by using an anti-apoB antibody to render the complexed apoB-containing fractions inaccessible to subsequently added cholesterol-measuring enzymes, followed by a standard enzymatic measurement of the level of HDL-C present, for which they provide premixed reagents. Typically, the HDL-C is measured by reacting the HDL-C with cholesterol esterase to liberate cholesterol ester bound to the HDL, and then reacting the now free cholesterol with cholesterol oxidase and oxygen to form hydrogen peroxide. The hydrogen peroxide can then be measured by a variety of means.

In Japanese Patent Kokai No. Hei 6-242110, lipoprotein fractions one does not want to measure are complexed with an appropriate antibody. Enzymes for

measuring cholesterol content are then added, and the cholesterol release and oxidation are permitted to proceed, and reagents are added to permit measurement of the hydrogen peroxide by colorimetric changes. To facilitate reading the colorimetric changes, which are impeded by turbidity caused by the presence of the antibody-antigen complex, a detergent is added to dissolve the complex. To prevent the measurement of the lipoprotein fraction being measured from being affected by the presence of the lipoprotein fraction being freed from the antibody-antigen complex, a heavy metal is added at the same time to poison the enzymes and terminate the enzymatic reaction.

Reagents other than antibodies have also been used to render non-HDL lipoproteins inaccessible to enzymatic reaction so that the HDL fraction can be measured. Roche Diagnostics (Basel, Switzerland), for example, provides a polyethylene glycol ("PEG") based system in which sulfated α-cyclodextrin, dextran sulfate and $MgCl_2$ form water soluble complexes with the non-HDL lipoproteins present in a sample, after which pegylated cholesterol esterase and cholesterol oxidase are introduced. The non-HDL complexes are not accessible to the PEG-modified enzymes, permitting measurement of the HDL fraction.

Another homogeneous assay is provided by Genzyme Diagnostics (San Carlos, CA and Cambridge MA), under the name Liquid N-geneous® HDL. In this method, synthetic polyanions adsorb to the surface of the non-HDL lipoproteins and render them unavailable to cholesterol esterase, while the HDL fraction is solubilized and undergoes conventional enzymatic reactions. The various homogenous assays for HDL are discussed and compared in, for example, Nauck et al., Clin Chem 44:1143-1451 (1998) and Harris et al., Clin Chem 43:816-823 (1997). The N-geneous® system is compared to phosphotungstic acid precipitation in, for example, Halloran et al., Arch Path Lab Med 123:317-326 (1999) and Hubbard et al., Am J Clin Path 110:495-502 (1998). The use of other polyanions to complex lipoprotein fractions is also known. See, e.g., Burstein et al., J. Lipid Res 11:583-595 (1970).

The reference method for determining LDL-C is the β-quantification method. This method arrives at an LDL-C measurement by a chemical precipitation using heparin and a divalent cation, such as manganese, magnesium or calcium and ultracentrifugation, which leaves the HDL fraction in the supernatant. The supernatant can then be removed and the LDL solubilized and reacted to determine the amount

present. LDL-C values have been also been obtained by the Friedewald calculation, once total-C, triglycerides, and HDL-C have been measured.

More recently, two homogeneous assays for LDL-C have been developed. One, introduced by Roche Diagnostics (Basel, Switzerland), employs reagents -- $MgCl_2$, sulfated α-cyclodextrin, and dextran sulfate -- to render the non-LDL fractions unavailable to the enzymes used in the enzymatic determinations. A non-ionic detergent is then introduced to selectively solubilize the LDL-C, which is then measured by conventional enzymatic reaction. In the second, commercially available from Genzyme (Cambridge, MA), a detergent selectively solubilizes the non-LDL fractions, which are then reacted with enzymes in the absence of a color label. After the non-LDL-C has been reacted, a second detergent solubilizes the LDL-C in the presence of a color substrate to permit conventional enzymatic measurement of the LDL-C. The relative merits and problems of these methods are reviewed and compared, for example, in Nauck and Rifai, Clinica Chimica Acta 294:77-92 (2000).

SUMMARY OF THE INVENTION

The invention relates to a method for performing a sequential homogeneous assay for cholesterol associated with lipoprotein fractions present in a sample. The method comprises, in the following order: contacting a first lipoprotein fraction in the sample with a complex-forming agent which selectively forms a complex with the first lipoprotein fraction to form an agent-first lipoprotein fraction complex, with the proviso that the complex is not a substrate for cholesterol esterase; measuring the amount of any cholesterol associated with a second lipoprotein fraction present in the sample by using cholesterol esterase and cholesterol oxidase to obtain a first cholesterol value; dissociating the first lipoprotein fraction from the first lipoprotein fraction/agent complex; and determining the total amount of cholesterol present in the sample. In some embodiments, the complex is also not a substrate for cholesterol oxidase and in others, it is not a substrate for cholesterol dehydroxgenase.

Further, by subtracting the value obtained for the first lipoprotein fraction from the total-C, the amount of the second lipoprotein fraction can also be determined. Thus, the methods of the invention can be used to determine HDL and total-C, allowing

calculation of the non-HDL-C in the sample, or to determine LDL-C and total-C, which allows calculation of the non-LDL-C in the sample.

The complex-forming agent can be, for example, an antibody which binds selectively to the first lipoprotein fraction, a polyanion, or a sulfated cyclodextrin. Suitable polyanions include heparin, dextran sulfate, phosphotungstic acid, polyvinyl sulfate, heparin sulfate, chondroitin sulfate, hyaluronic acid, and sulfated oligosaccharides.

In some embodiments, a non-denaturing detergent is used to dissociate the first lipoprotein fraction from the complex-forming agent. In a preferred embodiment, the detergent is deoxycholate.

The methods of the invention include measuring the amount of cholesterol present in the first and second lipoprotein fractions by reacting cholesterol ester in the fractions with cholesterol esterase, so that all the cholesterol in the lipoprotein fraction being measured is in the form of free cholesterol. Depending on the system chosen by the practitioner, the free cholesterol is then typically reacted with cholesterol oxidase or with cholesterol dehydrogenase. If desired, the value obtained for the first lipoprotein fraction is subtracted from the value obtained for the total cholesterol.

In some embodiments, the amount of the cholesterol present in the sample is determined by an optical means. In preferred embodiments, the optical means is a change in absorption or emission spectra of an indicator molecule. In some preferred embodiments, the indicator molecule is a dye. In some other preferred embodiments, the indicator molecule is NAD or NADP.

In yet another aspect of the invention, the method further comprises determining the amount of any triglycerides present in the sample.

In other aspects, the invention relates to kits for determining amounts of cholesterol present in a sample, comprising a complex forming agent, a non-denaturing detergent, and instructions for performing a method of the invention. The complex-forming agent can be, for example, an anti-apoB antibody or an anti-apoAI or anti-apoAII antibody. The complex-forming agent can alternatively be a synthetic polyanion or a sulfated cyclodextrin. The non-denaturing detergent can be, for example, deoxycholate. The kit can further comprise one or more enzymes useful in cholesterol or triglyceride measurements, such as lipase, glycerol kinase, glykinase, glycerol phosphate

dehydrogenase, glycerol phosphate oxidase, peroxidase, pyruvate kinase, and lactate dehydrogenase.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Diagram of DHT reaction profile.

Figure 2. Reaction profile of DHT assay. Panel A: HDL (50 mg/dL); Panel B: LDL (150 mg/dL); Panel C: HDL (50 mg/dL) plus LDL (150 mg/dL); Panel D: serum. Arrows indicate the point of adding the reagents for step 2 and step 3 for the DHT assay.

Figure 3. Lipoprotein cross reactivity of DHT assay. Panel A: HDL-C was fixed at 50 mg/dL and LDL-C was added at the indicated concentration shown on the X-axis. Measured values for HDL-C (O) in step 2 and LDL-C (Δ) in step 4 and total-C (□) are shown on the Y-axis. Panel B: LDL-C was fixed at 100 mg/dL and HDL-C was added at the indicated concentration shown on the X-axis. Measured values for HDL-C (O) in step 2 and LDL-C (Δ) in step 4 and total-C (□) are shown on the Y-axis. Results are shown as the mean plus and minus 1 S.D.

Figure 4. Linearity of HDL-C and Total-C for the DHT assay. Panel A: Linearity was determined by dilution of a concentrated sample of HDL and is shown as the measured HDL-C on the Y-axis versus the assigned concentrations of HDL-C on the X-axis. Panel B: Linearity was determined by dilution of a concentrated sample of LDL and is shown as the measured Total-C on the Y-axis versus the assigned concentrations of LDL-C on the X-axis. Results are shown as the mean plus and minus 1 S.D.

Figure 5. Precision of the DHT assay. Results for HDL-C and total-C are shown for within-run (n=20) and between-run (n=10) precision. Results are shown as the coefficient of variation (CV).

Figure 6. Comparison of DHT assay to standard assays. Panel A: HDL-C as determined by the DHT method (Y-axis) was compared to the results obtained by PolyMedco method (X-axis). Panel B: Total cholesterol as determined by the DHT method (Y-axis) was compared to the results obtained by Roche method (X-axis).

Panel C: Non-HDL-C as determined by the DHT method (Y-axis) was compared to the results obtained by subtraction of total cholesterol (Roche method) from HDL-C (PolyMedco method). (X-axis). Panel D: LDL-C calculated by the Friedewald equation using HDL-C and total cholesterol as determined by the DHT method (Y-axis) was compared to the calculated LDL-C using the standard assay for HDL-C (PolyMedco) and total cholesterol (Roche) (X-axis). Results are shown as the mean plus and minus 1 S.D.

DETAILED DESCRIPTION

I. Introduction

The invention provides new methods for determining the amounts of cholesterol associated with the various lipoprotein fractions present in a sample, such as a patient serum sample, in a series of simple steps. Although each of the steps is simple, in combination, they permit the determination of HDL-C and total-C, or of LDL-C and total-C, to be made from a single sample in a single tube in a single liquid phase. Optionally, the level of triglycerides present can also be determined. We initially the assay method to determine HDL and total cholesterol levels, and therefore dubbed the assay the dual HDL/total cholesterol, or "DHT" assay. For convenience, we continue to refer to our method by this name, even though it can also be used to determine LDL-C and total-C, and, with an extra step, triglycerides.

The standard DHT assay is performed in four steps, performed in order. In the first step, an agent is added which can complex selectively with a chosen lipoprotein fractions (the lipoprotein fraction which will be complexed with the complex-forming agent will be referred to hereafter as the "first lipoprotein fraction") which might be present in a sample. For example, an anti-apoB antibody can be added to complex with apoB-containing lipoproteins (LDL, VLDL, and chylomicrons) present in the sample, leaving the HDL-C lipoprotein uncomplexed. Or, an anti-apoAI or anti-apoAII antibody may be used to complex with apoAI- or apoAII-containing lipoproteins, such as HDL. The complex-forming agent renders the lipoprotein fraction with which it reacts unavailable to enzymatic reaction with cholesterol esterase and preferably at least one of the enzymes cholesterol oxidase or cholesterol dehydrogenase, which are used in common assays of cholesterol content.

In the second step, a measurement is made of the lipoprotein fraction which remains uncomplexed (the "second lipoprotein fraction"). Thus, in the example

just cited, if the complex-forming agent is an anti-apoB antibody, the HDL-C in the sample will remain uncomplexed and available for measurement. Typically, the measurement is performed by conventional enzymatic reactions. In one form of these conventional assays, the lipoprotein is first contacted with cholesterol esterase. The enzyme converts all the cholesterol in the lipoprotein, which is typically present as free cholesterol and as cholesterol ester, into free cholesterol. The free cholesterol is then reacted with cholesterol oxidase, in the presence of a reporter enzyme (typically peroxidase) which induces a colorimetric change of the indicator molecule in the presence of the product of the reaction with cholesterol oxidase. In a second conventional method, called the "NAD" method, following the reaction with cholesterol esterase, the cholesterol is reacted with cholesterol dehydroxgenase. This reduces NAD to NADH and results in a change in the absorption profile of the solution, which can be correlated to the cholesterol content. *See, e.g.*, Kayamoriy et al., Clin. Chem. 45:2158-63 (1999). In a variation of this method, the assay is performed utilizing NADP rather than NAD.

Cholesterol associated with the lipoproteins bound in the complex is sterically blocked from reacting with the enzymes of the method chosen and is not available as a substrate for the enzymes. The enzymes thus react with only the cholesterol associated with any HDL-C fraction present in the sample (if the non-HDL-C was complexed) or with any LDL-C present in the sample (if the HDL-C was complexed with the complex-forming agent).

Once the enzymes have effectively reacted fully with the cholesterol present in the second lipoprotein fraction (the fraction which was not complexed with the complex-forming agent) the third step is performed. In the third step, a detergent which can disrupt the agent-lipoprotein complex without denaturing the enzymes is added to the sample, enabling the enzymatic measurement of cholesterol associated with the lipoprotein fractions which were originally complexed with the agent (the first lipoprotein fraction). Since the enzymes have not been denatured, they are available to react with the cholesterol in the first lipoprotein fraction. Further, since all or substantially all of the cholesterol in the second lipoprotein fraction was reacted before addition of the detergent, any additional cholesterol which reacts with the enzymes after the addition of the detergent is considered to have come from the first lipoprotein fraction.

In the fourth step, the change in absorbance from the baseline in step 1 to the end of step 3 is measured. This change is proportional to total-C. The cholesterol in the first lipoprotein fractions (those which were originally complexed with the complex-

forming agent) can then be calculated by subtracting the results from the total cholesterol measurement (step 4) from the measurement of the second lipoprotein fraction (step 2). A diagram of the theoretical reaction profile of the DHT assay is shown in Fig. 1. It should be noted that, while the method has been described in four steps for convenience and clarity of presentation, in practice some of the steps can be combined and performed together. For example, steps 1 and 2, or steps 3 and 4 can be combined

By subtracting the value obtained for the first lipoprotein fraction from the total-C, the amount of the second lipoprotein fraction can also be determined. Thus, the methods of the invention can be used, for example, to determine HDL and total-C, allowing calculation of the LDL-C and VLDL-C in the sample, or to determine LDL-C and total-C, which allows calculation of the non-LDL-C in the sample. It should be noted that a calculation of non-LDL-C in the sample will combine the HDL-C in the sample (which is usually the next lipoprotein fraction of interest) with VLDL-C and other minor lipoprotein fractions. Similarly, when determining HDL-C by the methods of the invention, thus complexing the non-HDL-C, VLDL-C will generally be complexed with the LDL-C (which in this assay is the lipoprotein fraction of greatest interest next to the HDL-C).

Optionally, in yet another aspect, triglyceride levels can be measured. This is usually performed using lipase, glycerol phosphate dehydrogenase, glycerol phosphate oxidase, and peroxidase (which, like the cholesterol content assay using peroxidase, provides a colorimetric change) or, alternatively, by the "NAD" method, using lipase, glycerol kinase, pyruvate kinase, and lactate dehydrogenase (which, like the NAD method for determining cholesterol content, provides a change in the absorption profile of the solution, permitting correlation to the triglycerides present). Various conventional assays for measuring triglycerides are known in the art. For example, some nine commercially available assays are compared in Sampson et al., Clin. Chem. 40:221-226 (1994).

Measurement of the triglycerides present further extends the value of the assay. For example, if the method is used to measure HDL-C and then total-C and then triglycerides, LDL-C can be calculated by the Friedewald equation. Conversely, the method can be used to measure LDL-C and then total-C and, optionally, triglycerides, permitting non-LDL-C to be calculated by the Freidewald equation. Thus, the method is flexible and permits all the commonly measured lipoprotein fractions to be measured homogeneously in a single tube.

Based on the results obtained in numerous assays and comparisons to standard assays (such as those shown in Figures 2-6), the DHT assay has acceptable analytic performance and produces results similar to standard assays. But the DHT assay simplifies the approach for measuring the cholesterol content of lipoprotein fractions. Besides the DHT test, only an assay for total triglyceride is needed for determining the cholesterol content of lipoprotein fractions. Because the DHT test is a homogenous test, the assay can be performed in a single tube and no preprocessing steps, such as precipitation and centrifugation, are necessary. The DHT test is also cost effective, because no additional reporter enzymes are needed to measure total cholesterol once the HDL or LDL measurement has been made. Reducing the overall complexity and cost of performing lipoprotein fraction analysis is important because such analysis is widely used both for screening the population at risk for coronary artery disease and for guiding therapy. Moreover, the same test procedure can be used to assay either LDL-C or HDL-C. If the choice is to directly assay HDL, a complex-forming agent that complexes with LDL, such as an anti-apoB antibody, is used so that the LDL becomes the first lipoprotein fraction. If the choice is to directly assay LDL, a complex-forming agent that complexes with HDL, such as an anti-apoAI- or anti-apoAII antibody is used, so that the HDL in the sample becomes the first lipoprotein fraction.

In sum, the DHT test is a relatively simple method for the dual measurement of HDL-C and total cholesterol or of LDL-C and total cholesterol, and, if desired, of triglycerides. It provides a cost effective alternative for performing cholesterol lipoprotein fraction analysis. The DHT test is also flexible, allowing the use of different types of complex-forming agents. Examples 1 and 2, below, show the use of the invention using antibodies as the complex-forming agents. Examples 3 and 4 show that satisfactory results have been obtained using a synthetic polyanion and Example 5 shows that satisfactory results were obtained using a sulfated cyclodextrin as the complex-forming agent.

The discussion below provides further information regarding definitions of the terms used herein. It then proceeds to discuss complex-forming agents appropriate for use in the invention, detergents which can be used to disrupt the complex-forming agent-first lipoprotein fraction complex formed in step 1, systems for measuring the levels of cholesterol present in each lipoprotein fraction, and assays for measuring triglycerides as an optional series of steps in employing the inventive method.

II. Definitions

Unless defined otherwise, all scientific and technical terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any materials and methods similar to or equivalent to those described herein can be used in the practice of the invention, preferred methods and materials are described. For purposes of the present invention, the following terms are described below.

According to Stedman's Medical Dictionary (Hensyl, ed., 25th Ed., Williams & Wilkins, Baltimore MD, 1990) ("Stedman's"), a "lipoprotein" refers to a complex or compound containing lipid and protein. Stedman's notes that almost all the lipids in plasma are in the form of lipoproteins. *See*, Stedman's, at page 886, definition of "lipoprotein."

Stedman's classifies lipoproteins by their flotation constants, or densities ("d"), as follows: chylomicra, < 1.006; very low density lipoproteins ("VLDL"), 1.006-1.019; low density lipoproteins ("LDL"), 1.019-1.063; high density lipoproteins, 1.063-1.121, and very high density lipoproteins ("VHDL"), > 1.21. *Id.* Although interest has grown in the effect of the VHDL fraction in preventing arthlerosclerosis, VHDL is usually measured together with HDL. Unless otherwise specified or made clear in context, therefore, references herein to "HDL" also include any VHDL which may be present.

As used herein, "HDL" and "HDL-C" are generally used synonymously unless otherwise indicated or required by context.

As used herein, "LDL" and "LDL-C" are generally used synonymously unless otherwise indicated or required by context.

The term "associated with," means, with respect to the cholesterol content of a particular lipoprotein fraction, that the cholesterol is complexed with protein in particles classified, by their flotation constant, as belonging to the particular lipoprotein fraction under discussion.

The "cholesterol" in lipoproteins is present both as free cholesterol and as cholesterol ester. Typically, a lipoprotein is reacted with cholesterol esterase to convert the cholesterol ester in the lipoprotein to free cholesterol. All of the cholesterol in the lipoprotein is then available for further reactions. Unless otherwise indicated or required in context, references herein to "cholesterol" in a lipoprotein fraction refer to both free cholesterol and that present as cholesterol ester.

The term "complex-forming agent" (sometimes referred to simply as "agent") refers to a compound or molecule capable of selectively binding to a particular lipoprotein fraction, for example, to HDL or to LDL, to non-apoB containing lipoproteins, or to non-apoAI or non-apoAII containing lipoproteins. Frequently, the complex-forming agent is an antibody, such as an anti-apoB antibody. Polyanions and sulfated cyclodextrins can also be used to bind to a selected lipoprotein fraction. Preferably, the binding of the complex-forming agent does not alter or destroy the lipoprotein fraction to which it binds or complexes. Even more preferably, the agent sterically blocks the interaction of the lipoprotein to which it is complexed with cholesterol esterase, cholesterol oxidase, or both.

As used herein, a "non-denaturing detergent" is a detergent that (1) is able to dissolve the complex formed by a complex-forming agent, such as an antibody, and a lipoprotein fraction, and (2) does not inactivate cholesterol esterase, cholesterol oxidase, or significantly affect colorimetric measurement of the amount of cholesterol with standard reagents.

An "indicator molecule" refers to a molecule that can be used to monitor a reaction. Typically, this is done using a molecule such as a dye which changes its absorbance or emission spectra in the presence of the product of an enzymatic reaction of interest. In the case of a peroxidase-coupled reaction, the oxidation of the dye changes its absorption spectrum. In the case of the NAD and NADP reaction systems, the indicator molecule is the NAD or NADP, respectively, which change absorbance when reduced to NADH or NADPH, respectively.

As used herein, "determining" includes measuring, including by adding to or subtracting from a value to obtain a second value.

"Dissociating" means, with regard to a complex formed by a complex-forming agent and a lipoprotein fraction, to separate the complex, typically by disrupting or dissolving it.

An "anti-apoAI antibody" or "anti-apoAII antibody" means an antibody or antibody fragment which specifically recognizes and complexes with apolipoprotein AI or AII, respectively.

As used herein, an "anti-apoB antibody" means an antibody or antibody fragment which specifically recognizes and complexes with antigenic determinants on chylomicrons, VLDL, and LDL. Specifically, the term relates to antibodies which recognize apolipoprotein B.

An "anti-apoC antibody" is an antibody or antibody fragment which specifically recognizes apolipoprotein C. Similarly, an "anti-apoE antibody" is an antibody or antibody fragment which specifically recognizes apolipoprotein E.

"Specifically bind" means, with reference to a complex-forming agent such as an antibody, that the agent preferentially associates with a lipoprotein of the designated fraction and not at all, or in much smaller amounts, to other lipoprotein fractions. With respect to antibodies in particular, it is recognized that a certain degree of non-specific interaction may occur between a molecule and a non-target lipoprotein fraction. Nevertheless, specific binding may be distinguished as mediated through specific recognition of the antigen.

As used herein, an "antibody" refers to a protein functionally defined as a binding protein and structurally defined as comprising an amino acid sequence that is recognized by one of skill as being derived from the framework region of an immunoglobulin encoding gene of an animal producing antibodies. An antibody can consist of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively.

Antibodies exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'2, a dimer of Fab which itself is a light chain joined to VH-CH1 by a disulfide bond. The F(ab)'2 may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the (Fab')2 dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see*, Fundamental Immunology, W.E. Paul, *ed.*, Raven Press, N.Y. (1993), for a more detailed description of

other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies, and also includes single chain antibodies (antibodies that exist as a single polypeptide chain). The term also encompasses single chain Fv antibodies (sFv or scFv) in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide. The single chain Fv antibody is a covalently linked VH-VL heterodimer which may be expressed from a nucleic acid including VH- and VL- encoding sequences either joined directly or joined by a peptide-encoding linker. Huston, et al. (1988) Proc. Nat. Acad. Sci. USA, 85: 5879-5883. While the VH and VL are connected to each as a single polypeptide chain, the VH and VL domains associate non-covalently. Preferred antibodies include scFv, Fv, Fab and disulfide linked Fv (Reiter et al. (1995) Protein Eng. 8: 1323-1331). Antibodies can also include diantibodies and miniantibodies.

Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art. *See, e.g.*, Coligan (1991), CURRENT PROTOCOLS IN IMMUNOLOGY, Wiley/Greene, NY; and Harlow and Lane; Stites *et al.* (eds.) BASIC AND CLINICAL IMMUNOLOGY (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding (1986), MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975), *Nature*, 256:495-497. Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors. *See*, Huse *et al.* (1989), *Science*, 246:1275-1281; and Ward *et al.* (1989), *Nature*, 341:544-546.

III. Complex-Forming Agents

Complex-forming agents suitable for use in the present invention are those which specifically recognize structural determinants on lipoprotein fractions other than the one to be measured in a particular assay and sterically interfere with access by cholesterol esterase and typically of cholesterol oxidase to the complex. Further, the complex formed between the complex-forming agent and the lipoprotein fraction with which it complexes should be capable of disruption and the agent should not destroy or cleave the lipoprotein or otherwise act on the lipoprotein in a manner which prevents the

lipoprotein from reacting with cholesterol esterase or cholesterol oxidase once the complex between the agent and the lipoprotein is disrupted.

In one important set of preferred embodiments, the complex-forming agents are antibodies. Anti-apoB antibodies constitute one important set of antibodies useful in the present invention. Apolipoprotein B, or "apoB," is associated with chylomicrons, VLDL, and LDL; thus, an anti-apoB antibody added to a sample complexes with all the non-HDL lipoprotein fractions. Low density lipoproteins are now considered by some in the art to comprise a group of related lipoproteins which can be differentiated by their associated proteins.

Anti-apoAI and anti-apoAII antibodies constitute a second important set of antibodies useful in the present invention. ApoAI constitutes about 75-80% of HDL lipoprotein and is found on all HDL species. Accordingly, anti-apoAI antibodies can be used alone to obtain a good approximation of LDL and HDL levels. For more accurate readings, anti-apoAII antibodies can be used. Apolipoprotein AII constitutes about 20% of total HDL protein. It is also present on a small percentage of LDL species; it is anticipated however, that complexing with this small percentage of the overall LDL present in a sample would not significantly affect the overall accuracy of the test and would not, for example, bring its overall accuracy of measuring HDL below the level set as acceptable by the National Cholesterol Education Program (*see, e.g.*, Warnick and Wood, Clin. Chem. 41:1427-33 (1995). Thus, anti-apoAI and anti-apoAII antibodies complex with the HDL fraction and are used when the desire is to measure LDL-C and total-C using the methods herein.

While the invention will be most often be used to determine HDL, LDL, and total-C levels present in a sample, it is occasionally desirable to determine the amount of particular subfractions of lipoproteins present. Such subfractions can be determined using the method of the invention, using a complex-forming agent that complexes with the lipoprotein fraction of interest. For example, antibodies which recognize apoE, apoC, or apolipoprotein J can be used to determine the amounts of lipoprotein present bearing those determinants.

Use of the antibodies results in the formation of an antibody/lipoprotein complex which sterically blocks interaction of the complexed lipoproteins with the enzymes used to measure cholesterol, typically cholesterol esterase and cholesterol oxidase. The cholesterol in the lipoprotein fraction which has not been complexed is then measured, until all the cholesterol available in the non-complexed fraction has been

reacted. The complex is then dissolved, freeing the previously-complexed lipoproteins to be available as substrates for the enzymes and the amount of cholesterol freed can then be measured.

In addition to antibodies, synthetic polymers, in particular, polyanions, can be used as complex-forming agents. In a preferred embodiment, the synthetic polyanion supplied by Genzyme in its N-geneous® HDL test kit has proven satisfactory in the methods of the invention. A number of polyanions are known in the art, such as heparin, dextran sulfate, phosphotungstic acid, and polyvinyl sulfate, as well as divalent cations, such as calcium, magnesium and manganese chloride. See, e.g., Kerscher, U.S. Patent No. 4,746,605 (which for example sets forth molecular weights and preferred concentration ranges); Karl, U.S. Patent No. 5,804,450 (which for example sets forth additional polyanions); and Hino, U.S. Patent No. 5,773,304 (which for example sets forth use of polyanions or divalent cations in the presence of surfactants). Miki, U.S. Patent No. 5,925,534, also lists sulfated cyclodextrin, heparan sulfate, chondroitin sulfate, hyaluronic acid, sulfated oligosaccharides, sulfated polyacrilymides, carboxymethylated polyacrylamides and salts of these as polyanions, although it states that the better known polyanions heparin, phosphotungstic acid and dextran sulfate and salts thereof are preferred. It indicates that the concentrations of the polyanion in a reagent to be mixed with a patient sample is usually 0.0001% to 10 % (w/v) and is preferably 0.001 % to 1 % (w/v). It further states the polyanions can be used singly or in combination.

The '534 patent lists sulfated cyclodextrin as a polyanion, presumably because of the charge conferred by the sulfate groups. Unsulfated cyclodextrins complex with lipoproteins, however, sulfated cyclodextrins complex more selectively due to their charge. As discussed further in Example 5, below, DHT assays performed using α-cyclodextrin and the other reagents in the Roche LDL-C measuring system successfully determined both LDL-C and total-C, permitting the calculation of non-LDL-C, and compared well to standard assays measuring just LDL-C or just total-C.

The suitability of any particular complex-forming agent, such as a cyclodextrin, a polyanion, or an anti-apoB antibody, to block the reactivity of cholesterol esterase and cholesterol oxidase with the complexed lipoprotein fraction and the amount to use can be determined by performing "ranging" assays using a known amount of a purified lipoprotein fraction of the type to which the particular complex-forming agent binds. For this purpose, a cholesterol assay can be performed, as described below, without first adding a complex-forming agent normally added in step 1. The cholesterol

measurement resulting from the ranging assay should represent the total cholesterol content of LDL. Thereafter, a series of further assays can be performed, in which each successive assay contains an amount of the complex-forming agent which is greater than that used in the preceding assay. If the complex-forming agent is forming a complex and hindering access of the agent to the This should result in a successively decreasing amount of cholesterol measured.

The amount of complex-forming agent to be used should be sufficient to completely block the detection of cholesterol from the lipoprotein fraction to which it is intended to complex. The series of assays described above can provide this value. To ensure that the amount of antibody used is sufficient, the concentration of the lipoprotein used in the ranging assays described above should be in the upper range of the concentrations of that lipoprotein likely to be encountered. The normal ranges for each lipoprotein fraction, and the abnormal values reached in individuals with pathological conditions or unusual genetic conditions is known in the art. For example, with respect to LDL-C, a value of approximately 500 mg/dL is sufficient.

IV. Enzymes

Enzymes used in the DHT assay include cholesterol esterase, cholesterol oxidase, peroxidase; enzymes used in the triglyceride assay include lipase, glycerol kinase, glycerol phosphate dehydrogenase, and glycerol phosphate oxidase. These enzymes are commercially available from a number of suppliers, including Sigma Diagnostics (St. Louis, MO), Roche Molecular Biochemicals (Indianapolis, IN), Wako Chemicals (Richmond, VA), and ICN Biomedicals (Costa Mesa, CA). Most or all of the commercially sold enzymes are of bacterial source. Cholesterol esterase ("CHE") and lipase from *Pseudomonas,* cholesterol oxidase ("CO") from *Nocardia,* and glycerol kinase from *Candida* in the assays have proven satisfactory. The glycerol phosphate oxidase is, in standard reference, E.C. 1.1.3.21.

While a problem in conducting the assays with enzymes from any particular source has not been noted, if desired, an enzyme can be tested for its suitability for use in the assays set forth herein, such as the DHT assay, or the DHT and triglyceride assay, by running the assays on a known concentration of cholesterol, of triglycerides, or of both, and determining whether the results of the assay are within an acceptable range of the known value. Reagents containing known amounts of cholesterol and of

triglycerides are available from a number of sources, including Roche and Sigma Diagnostics.

In Examples 1 and 2, below, the reagents used in steps 1 and 2 of the assay are Reagents 1 and 2 of the EZ-HDL™ cholesterol measurement kit from Sigma Diagnostics. If desired, however, the reagents contained in this kit (anti-human apoB antibody and the reporter compounds peroxidase (POD) and 4-aminoantipyrine ("4AA") in the case of Reagent 1 and CHE and CO and FDAOS (N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxy-4-fluoroanaline, sodium salt) in the case of Reagent 2) can be added separately. Determination of the amount of antibody to be used can be determined by the assays described in the preceding section. Appropriate amounts of the reporter compounds and enzymes can be determined by performing a series of test assays on commercially available reagents containing known concentrations of lipoprotein fractions. Examples 3, 4, and 5, report the results of assays using different complex-forming agents to measure HDL-C and total-C or LDL-C and total-C. The enzymes used in these assays were from the same sources as those noted above.

Triglyceride measurements are most commonly made using glycerol kinase, glycerol phosphodehydrogenase, glycerol phosphate oxidase, and peroxidase. Other enzymatic assays for measuring triglycerides can, however, be incorporated into the methods taught herein. For example the "NAD" method uses glycerol kinase, pyruvate kinase and lactate dehydrogenase to measure triglycerides. All of these enzymes are also commercially available from Sigma and other suppliers.

V. Detergents

Following measurement of the uncomplexed lipoprotein component of the sample, the complex between the complex-forming agent and the complexed lipoprotein fraction is treated to separate the two. For purposes of the methods taught herein, the complex is preferably separated by dissolving the complex with a non-denaturing detergent. The detergent should be strong enough to dissolve the complex, but gentle enough not to denature the enzymes used during the course of the lipoprotein or triglyceride assays. A non-denaturing detergent suitable for use in the invention is therefore one which (1) dissolves the complex formed between the complex-forming agent and the complexed lipoprotein fraction and (2) does not inactivate cholesterol esterase, cholesterol oxidase, or peroxidase and does not interfere with the measurement

of the amount of cholesterol present. The strength of a detergent can be measured and characterized by, for example, its hydrophilic lipophilic balance, as taught in U.S. Patent No. 5,766,629.

Detergents preferred for use in the invention are deoxycholate, NP-40, and octyl glucoside, with deoxycholate being the most preferred. Any non-denaturing detergent meeting the test above, however, can be used. Any particular detergent can be readily tested to determine whether it is satisfactory for use in the invention. For example, a sample containing known concentrations of HDL and LDL can be tested by complexing the LDL component with anti-apoB antibodies, reacting the HDL with CHE and CO, and then using the detergent under consideration. If the resulting measurement of the LDL component is within an acceptable range of the known concentration, then the detergent has successfully dissolved the complex-forming agent/lipoprotein complex and has not impaired the measurement. A reading well below the known amount of LDL present would indicate that the detergent has failed one of the prongs of the test and is not satisfactory for use. Conveniently, the detergent is tested on a portion of a sample assayed in parallel with a second portion of the sample tested with deoxycholate as the detergent to verify that any the problem is with the detergent under consideration and not with the equipment or with an experimental error. As previously noted, reagents containing known concentrations of cholesterol (sometimes known in the art as cholesterol calibrators) are commercially available from several sources, including Roche and Sigma Diagnostics.

VI. Optical measuring systems

In preferred embodiments, the amount of cholesterol present is determined by optical means. In colorimetric assays, reagents are used to permit a measurement of the amount of light absorbed by a dye which undergoes a color change calibrated to the amount of substrate (ultimately, cholesterol) present in the sample. In fluorescent assays, a reagent is added which is excited by a light and which emits light of a different color, the amount of which is calibrated to the amount of substrate, and ultimately, the amount of cholesterol present. In chemiluminescent assays, light is spontaneously emitted by the reagents in response to the amount of substrate, permitting a measure of cholesterol present.

In a particularly preferred embodiment, chromogenic systems are used in which the amount of cholesterol present is determined by changes in the absorbance of

the sample solution as calibrated to the amount of light reaching a detector at 600 nm. Typically, cholesterol esterase and cholesterol oxidase react with cholesterol in the presence of H_2O and O_2 to form cholestenone, fatty acid and H_2O_2. Chromogenic systems which can then be used to detect the presence of H_2O_2, such as 4-aminoantipyrine ("4AA") and 3-methyl-2-benzothiazolinone hydrazone (MBTH) or its sulphonated derivative, are well known. *See, e.g.*, U.S. Patent No. 4,851,335. Additional reagents, such as FDAOS (N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxy-4-fluoroanaline, sodium salt), are often included in commercial reagent kits to speed the reaction.

In NAD and NADP systems, the reduction of NAD or NADP molecules, respectively, present in a solution containing a sample causes a change in the absorbance profile of the solution (typically measured at 340 nm). This absorbance change can be correlated to the amount of cholesterol or of triglycerides present in the sample, and does not require the presence of a dye.

VII. Triglyceride measurement

Triglycerides are comprised of fatty acid molecules bound to a glycerol backbone. *See, e.g.*, Stryer, *Biochemistry*, W. H. Freeman and Co. New York (3rd Ed. 1988), Chapter 20. In the classic enzymatic reaction for measuring triglyceride levels, lipase is used to cleave the fatty acids from their glycerol backbones. ATP is added, and glycerol kinase is used to phosphorylate the glycerol. Glycerol phosphate oxidase then generates glycerol and H_2O_2. An alternative assay format uses lipase, glycerol kinase, pyruvate kinase, and lactate dehydrogenase. *See, e.g.*, Sampson et al., Clin. Chem. 40:221-226 (1994). Triglyceride measurements can be made following step 4 of the method of the invention. When using the classic reaction with glycerol phosphate oxidase, the H_2O_2 formed by the reaction can be detected by the chromogenic systems already in the tube in connection with the lipoprotein fraction measurements, as discussed in the previous section.

Reagent kits containing enzymes for triglyceride measurements, along with reporter compounds, are commercially available from a number of suppliers, such as Sigma and Wako.

VIII. Modifications to Automated Assay Formats

Some automated assay devices, such as the Genzyme N-geneous® HDL system, are designed for the addition of only two reagents. Reagent 1 contains the polyanion, and reagent 2 contains the cholesterol esterase, cholesterol oxidase, and other enzymatic reagents used for the colorimetric measurement of cholesterol content in the HDL fraction. The methods of the invention can be practiced in such systems by combining the polyanion and the enzymes into a single reagent so that the detergent can be added as the second reagent. An exemplary assay as performed in our laboratory is set forth in the Examples.

Automated systems which permit the addition of more than two reagents should be usable in the methods of the invention without modification. If the system permits only two reagents to be added, and uses a complex-forming agent other than a polyanion, a test should be performed to see if the complex-forming agent and the enzymes used for the release and measurement of cholesterol can be combined without significantly affecting the results. This can be easily accomplished by taking a sample containing of a known amount of LDL-C or of HDL-C and dividing it into two portions. One portion is assayed in the system following the standard methodology for that system, and the second portion is tested in the system with the reagents provided in the system combined into a single reagent and the detergent added as a second reagent. A difference in the results of the two assays which is greater than the difference permitted by the standard deviation of the system as determined by the manufacturer indicates that the reagents supplied by the system cannot be combined into single reagent for use in the assays taught herein.

IX. Kits

The reagents necessary or useful for practicing the methods of the invention can be conveniently provided as kits. Typically, the kits will provide a container containing one or more complex-forming agents, such as an anti-apoB or an anti-apoAI antibody, a cyclodextrin, or a polyanion. The kit may further provide a container holding a detergent, such as deoxycholate. The kit may further provide containers holding one or more enzymes for enzymatic determination of cholesterol, such as cholesterol esterase, cholesterol oxidase, and may also provide reporter dyes to detect the formation of hydrogen peroxide generated by the action of cholesterol oxidase on cholesterol released from a lipoprotein fraction by cholesterol esterase.

In addition, the kit may provide containers holding one or enzymes for the measurement of triglycerides. For the standard assay, these enzymes may include lipase, glycerol kinase, glycerol phosphate dehydrogenase, glycerol phosphate oxidase, and peroxidase. Alternatively, or in addition, the kit may provide enzymes useful in the NAD method of measuring triglycerides, specifically, lipase, glycerol kinase, pyruvate kinase, and lactate dehydrogenase.

EXAMPLES

Example 1

This example demonstrates use of a method of the invention to determine the HDL-C and total cholesterol content of a sample.

Materials and Methods

HDL (d=1.063-1.21 g/dL) and LDL (d=1.009-1.063 g/dL) were obtained by density gradient ultracentrifugation, as previously described (Schumaker, V.N. *et al., Academic Press Inc., London* **128**:155-169 (1986)). Deoxycholate and a homogenous HDL-cholesterol kit (EZ-HDL™) were obtained from Sigma Diagnostics (St. Louis, MO). Cholesterol calibrators and reagents for total cholesterol were obtained from Roche (Indianapolis, IN) and performed on a Hitachi 917 analyzer (Roche). The HDL precipitation method was performed with reagents from PolyMedco (Cortland Manor, NY) and cholesterol was measured on a Cobas Fara analyzer using reagents from Roche.

The DHT test was performed on a Cobas Fara II analyzer (Roche) by modifying the Sigma homogenous HDL cholesterol assay kit, using the parameters shown in Table 1. HDL-C was calibrated using Preciset cholesterol calibrators (Roche) at 3 levels (50, 200, and 400 mg/dL). Total-C was calibrated by taking the factor (slope) generated by the Cobas step 2 and applying it to the raw data generated during step 3.

Table 1. DHT Assay Parameters

STEP	VOLUME	TIME
Step 1		
Add sample	2.5 μL	
Add antibody	225 μL	4 min

Step 2		
Add CE, CO (enzymatic reagents for cholesterol determination)	75 µL	5 min
Read absorbance.		
Step 3		
Add 100 mmol/L DOC	15µL	5 min
Step 4		
Note: enzymatic reagents for cholesterol measurement are already present from Step 2		Read absorbance after incubation with detergent

Legend: CE = Cholesterol esterase, CO = Cholesterol oxidase, DOC = Deoxycholate (Note: this legend is also followed in the Tables below.)

Results

The reaction profile of the DHT assay for purified fractions of HDL, LDL, and serum is shown in Fig. 2. In each panel of Figure 2, the arrow for "Step 2" represents a time point at which enzymes have been added to a mixture of sample and Anti-apoB antibody. In each panel, "Step 3" represents a time point at which detergent has been added and further colorimetric measurements made. As shown in Figure 2A, HDL-C is detected in only the second step, whereas Fig. 2B shows LDL-C was detected only after the addition of detergent in the third step. When a mixture of HDL and LDL was analyzed, cholesterol was detected in both steps 2 and 3 (Fig. 2C). Likewise, a positive reaction for cholesterol was detected in both steps 2 and 3 when serum was analyzed (Fig. 2D). The enzymatic reactions for detecting cholesterol in each step at 37°C were complete after approximately 5 minutes.

The cross reactivity of HDL-C and LDL-C during each step of the DHT reaction was demonstrated using purified HDL and LDL fractions (see Fig. 3). In Fig 3A, a fixed concentration of HDL-C was measured in the presence of an increasing amount of LDL-C (shown on the X-axis). In Fig 3B, a fixed concentration of LDL-C was measured in the presence of an increasing amount of HDL-C (shown on the X-axis). As can be

seen in Fig. 3A, the concentration of HDL-C when measured in step 2 did not significantly change as the amount of LDL-C in the sample was increased. The increasing amount of LDL-C was not detected during step 2 but was measured in step 4. Similarly, increasing amounts of HDL-C did not significantly affect the measurement of LDL-C in step 3 (Fig. 3B). The sum of HDL-C and non-HDL-C was equal to the total-C measurement as determined by the overall change in absorbance from step 1 to step 4.

The linearity of HDL-C and total cholesterol for the DHT assay is shown in Fig. 4. Both the HDL-C and total cholesterol portions of the assay were linear throughout the typical range of serum HDL-C and total cholesterol concentration. The within-run and between-run precision for the DHT assay is shown in Fig. 5 for a frozen serum pool. Both the HDL-C and total cholesterol portions of the assay showed relatively good precision compared to other HDL-C and total cholesterol assays.

The measurement of HDL-C and total cholesterol as determined by the DHT assay was compared to standard assays on sera from patients with a broad range of lipoprotein values (Fig. 6). Both the HDL-C and total cholesterol portions of the assay compared favorably to the standard assays. In addition, the calculated non-HDL cholesterol and the calculated LDL-C by the DHT assay also showed a close correlation with the standard assays.

Discussion

Total cholesterol in serum is typically measured enzymatically using cholesterol esterase and cholesterol oxidase. Cholesterol esterase ("CE" or "CHE") converts cholesterol ester to free cholesterol. The free cholesterol is then oxidized by cholesterol oxidase ("CO"), which generates a molecule of hydrogen peroxide. The generation of hydrogen peroxide is then detected by the oxidation of various dyes by peroxidase. The oxidation of the reporter dye results in a change of its absorption spectrum, which is used to determine the concentration of total cholesterol. Assays for total cholesterol typically contain a detergent, which does not adversely affect the activity of cholesterol esterase and cholesterol oxidase but accelerates the rate of the reaction by increasing the substrate availability of cholesterol esterase and cholesterol oxidase. The detergent disrupts and solubilizes the lipoproteins into smaller micelles, thus increasing the surface area of the lipid interface and increasing the rate of reaction of cholesterol

esterase and cholesterol oxidase. Addition of detergent at the beginning of the present procedure, however, would impede the ability to measure HDL-C in the first measurement. Accordingly, in the present methods, the detergent is not added until after the HDL-C reading is taken.

Homogenous assays for HDL-C use various strategies for blocking the reactivity of cholesterol esterase and cholesterol oxidase to the apoB containing lipoproteins (Nauck M. *et al., Clin Chem* 44:144-51 (1998)). The original homogenous HDL-C assay that was modified in creating the DHT assay uses an antibody directed against apoB to sterically block the reactivity of the apoB containing lipoproteins to cholesterol esterase and cholesterol oxidase. The assay is performed in the absence of detergent in order to prevent any disruption in the interaction of the antibody to the apoB containing lipoproteins. In the DHT assay of the invention, deoxycholate, a non-denaturing detergent is added after the measurement of HDL-C is complete in step 2. As can be seen in Fig. 2, after approximately 5 minutes, the reaction of cholesterol esterase and cholesterol oxidase with HDL is complete. This is not because the enzymes are no longer active or because of the consumption of the unoxidized dye but because of the enzymes have depleted the cholesterol on the HDL present in the sample. The addition of deoxycholate disrupts the antibody-apoB complex, which results in an additional increase in absorbance from the enzymatic reaction of cholesterol on the apoB containing lipoproteins.

Example 2

This Example describes coupling the DHT assay to an assay for triglycerides. It sets forth the standard protocol we employ for the DHT assay, as well as the modifications employed to accommodate the triglyceride assay.

The DHT assay and the triglyceride assay can be performed in sequence on a Cobas Fara II analyzer. Due to limitations on the sample volume that can be handled by the automatic pipetter of the analyzer, and to constraints on the ability of the device to read the absorbance of the dye above certain concentration levels, when a triglyceride assay will be performed following the DHT assay, we have found it useful to make a 10 fold dilution of the sample (25 μL serum + 225 μL of saline). The analyzer is then set to pipette the sample at 12.5 μL (which is 5 times the volume -- 2.5 μL -- used when only

the DHT assay is being performed), so that the final dilution of the sample is half the concentration used for the DHT assay, along with 225 µL of Reagent I of the EZ-HDL™ cholesterol measurement kit (Sigma Diagnostics), which is a premixed solution of the appropriate compounds.

After a four minute incubation and the first reading, the analyzer adds 75 µL of Reagent 2 (Sigma Diagnostics EZ-HDL™ kit), which contains CHE and CO and FDAOS. After a five minute incubation, reading 2 is taken, and then 15 µL of 100 mmol/L deoxycholate is added. After a five minute incubation, reading 3 is taken, and 50 µL of a mixture of lipase (2500 U/L), glycerol kinase (1250 U/L), glycerol phosphate dehydroxgenase (2000 U/L), and glycerol phosphate oxidase (2500 U/L), is added which contains the enzymes needed for the triglyceride measurement with the GPO Trinder reaction. It should be noted that the 4AA, POD, and FDAOS added for the first reading are still in the sample mixture and act to permit the colorimetric determination of the level of triglycerides present, expressed in mg/dL.

For the readings set forth in the Figures, the amounts of the various lipoprotein fractions were determined by readings of absorbance taken at 600 nm on a device calibrated by the absorbance of known amounts of cholesterol and triglycerides.

Example 3

In this Example, compatibility of the invention with the Genzyme N-geneous® HDL measurement kit was determined. The N-geneous® kit was used according to the manufacturing directions to perform steps 1 and 2 of the invention; that is, to complex a lipoprotein fraction (in the case of the N-geneous® kit, the LDL fraction) and to obtain a HDL reading. The remaining steps of the method were then performed. The procedure is set forth in the following Table:

Table 2.

STEP	VOLUME	TIME	RESULT
Step 1			
Add patient or other sample	2.5 µL		
Add Genzyme Reagent 1 (polyanion)	250 µL	5 min	

Step 2			
Add Genzyme Reagent 2 (CE, CO, enzymatic reagents for cholesterol determination)	85μL	5 min	HDL-C
Read absorbance			
Step 3			
Add 100 mmol/L DOC	15 μL	5 min	
Step 4			
Read Total-C absorbance following incubation with detergent. (Enzymatic reagents are already present from Step 2.)			Total-C

Results.

The Genzyme N-geneous® system was completely compatible with the methods of the invention. The synthetic polyanion used to complex with the LDL was readily solubilized with the detergent, deoxycholate, which permitted the reading of total-C. Non-HDL-C can then be calculated, if desired, by subtracting the HDL-C value from the total-C reading obtained in step 4.

Example 4

In this Example, the Genzyme N-geneous® HDL-C measuring system was modified. The system is designed for the addition of two reagents. Since the method of the invention employs a detergent as an additional reagent, the reagents used in the Genzyme system were combined so that the detergent could be added as the second reagent. The modified system was tested, as shown in the following table:

Table 3.

STEP	VOLUME	TIME	RESULT
Steps 1 and 2			
Add sample	2.4 µL		
(Optional: read baseline absorbance)			
Add combination of Genzyme Reagent 1 (polyanion) and Genzyme Reagent 2 (CE, CO, enzymatic reagents for cholesterol determination)	320 µL	5 min	HDL -C
Read absorbance			
Step 3			
Add 100 mmol/L DOC	15 µL	5 min	
Step 4			
Read absorbance following incubation with detergent.			Total-C
(Enzymatic reagents are already present from Step 2.)			

Results.

The modified Genzyme N-geneous® system was completely compatible with the methods of the invention. The combined reagents (synthetic polyanion used to complex with the LDL and the enzymes for determining HDL-C) could be added together and the LDL-polyanion complex was readily solubilized with the detergent, deoxycholate, which permitted the reading of total-C. Non-HDL-C can then be calculated, if desired, by subtracting the HDL-C value obtained from the total-C reading obtained in step 4.

Example 5

This Example shows the use of the assays of the invention to determine LDL-C. The Roche LDL-C assay uses sulfated α-cyclodextrin to complex apoB-

containing lipoproteins. The assay was performed using the Roche reagents in a Cobas Fara II analyzer.

The assay procedure is set forth in the following Table:

Table 4. Using Roche LDL-C reagents to determine LDL + TC in the DHT assay.

STEP	VOLUME	TIME	RESULT
Step 1			
Add sample	3.0 µL		
Add Roche Reagent 1 (α-Cyclodextrin, buffer)	225 µL	5 min	
Read baseline absorbance			
Step 2			
Add CE, CO (enzymatic reagents for cholesterol determination)	75µL	5 min	LDL -C
Read absorbance			
Step 3			
Add 100 mmol/L DOC	15 µL	10 min	
Step 4			
Read absorbance (Enzymatic necessary for reading are present from Step 2).			Total-C

Tests on a number of patient samples showed satisfactory correspondence between the LDL and Total-C measurements obtained by the DHT assay and the measurements obtained using standard tests for LDL-C and for Total-C.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for determining amounts of cholesterol in lipoprotein fractions present in a sample, comprising the following steps, in the following order:

(a) contacting a first lipoprotein fraction in the sample with a complex-forming agent to form a complex of said first lipoprotein fraction with the complex-forming agent, with the proviso that the complex is not a substrate for cholesterol esterase;

(b) measuring the amount of cholesterol associated with a second lipoprotein fraction present in the sample, to obtain a first cholesterol value;

(c) dissociating the first lipoprotein fraction from the complex-forming agent; and,

(d) measuring the total amount of cholesterol present in the sample, thus determining the amount of cholesterol in the first and second lipoprotein fractions present in the sample.

2. A method of claim 1, further provided that the complex of step (a) is not a substrate for cholesterol oxidase.

3. A method of claim 1, further provided that the complex of step (a) is not a substrate for cholesterol dehydrogenase.

4. A method of claim 1, wherein said first lipoprotein fraction is HDL-C and said second lipoprotein fraction is non-HDL-C.

5. A method of claim 1, wherein said first lipoprotein fraction is LDL-C and said second lipoprotein fraction is non-LDL-C

6. A method of claim 1, wherein said complex-forming agent is selected from the group consisting of: an antibody which binds specifically to lipoproteins of said first lipoprotein fraction, a polyanion, and a sulfated cyclodextrin.

7. A method of claim 6, wherein said polyanion is selected from the group consisting of: heparin, dextran sulfate, phosphotungstic acid, polyvinyl sulfate, heparin sulfate, chondroitin sulfate, hyaluronic acid, and a sulfated oligosaccharide.

8. A method of claim 1, wherein said first lipoprotein is dissociated from the complex-forming agent by a non-denaturing detergent.

9. The method of claim 8, wherein the detergent is deoxycholate

10. A method of claim 1, wherein the measuring of the amount of cholesterol present in steps (b) and (d) is performed by reacting cholesterol ester in the sample with cholesterol esterase.

11. A method of claim 10, wherein said cholesterol is reacted with cholesterol oxidase or cholesterol dehydrogenase.

12. The method of claim 1, further wherein the first cholesterol value is subtracted from the total amount of cholesterol.

13. The method of claim 1, wherein the amount of the lipoprotein present in the sample is determined by an optical means.

14. The method of claim 13, wherein the optical means is a change in absorption or emission spectra of an indicator molecule.

15. The method of claim 14, wherein said indicator molecule is a dye.

16. The method of claim 1, further comprising determining the amount of any triglycerides present in the sample.

17. A method of claim 1, wherein said first lipoprotein fraction consists of any apoB-containing lipoproteins in the sample.

18. The method of claim 17, further wherein said complex-forming agent is an anti-apoB antibody.

19. The method of claim 1, wherein said first lipoprotein fraction consists of any HDL-C present in the sample.

20. The method of claim 19, further wherein said complex-forming agent is an antibody which specifically binds to HDL lipoproteins.

21. The method of claim 20, wherein said antibody specifically binds to apoAI or apoAII.

22. A kit for determining amounts of cholesterol present in a sample, comprising a complex-forming agent and a non-denaturing detergent.

23. A kit of claim 22, further comprising one or more enzymes selected from the group consisting of cholesterol esterase, cholesterol oxidase, and cholesterol dehydrogenase.

24. A kit of claim 22, wherein the complex-forming agent is an anti-apoB antibody.

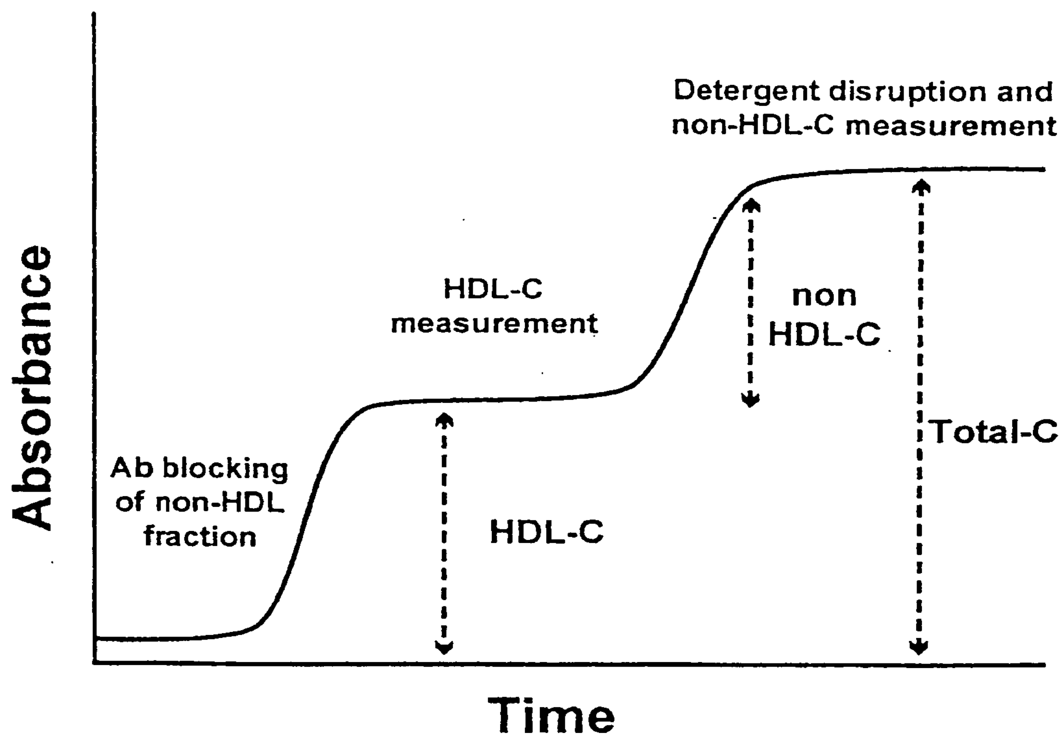
25. A kit of claim 22, wherein the complex-forming agent is an anti-apoAI or anti-apoAII antibody.

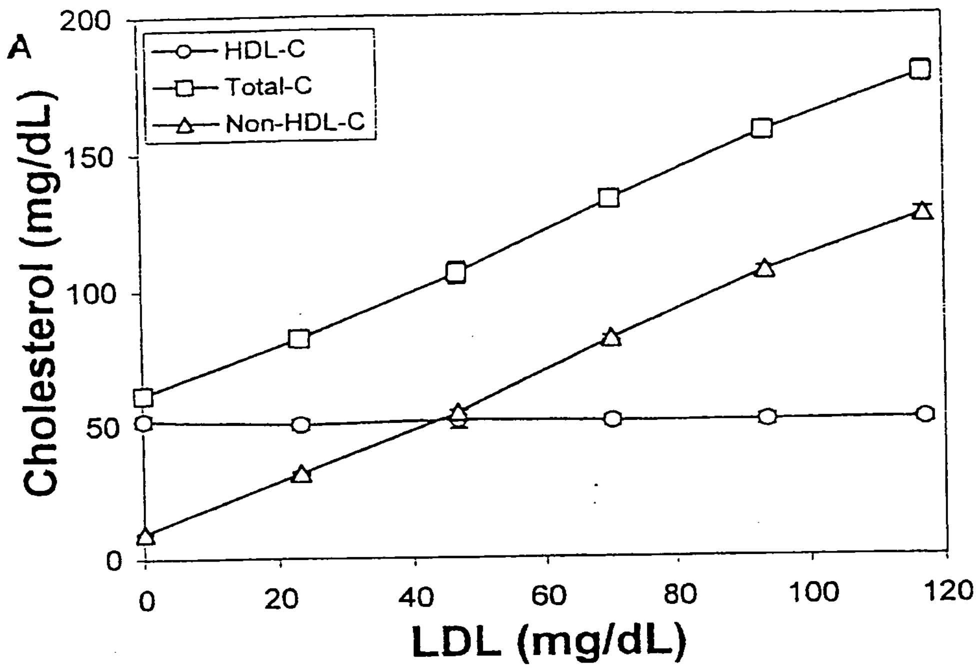
26. A kit of claim 22, wherein the complex-forming agent is a synthetic polyanion.

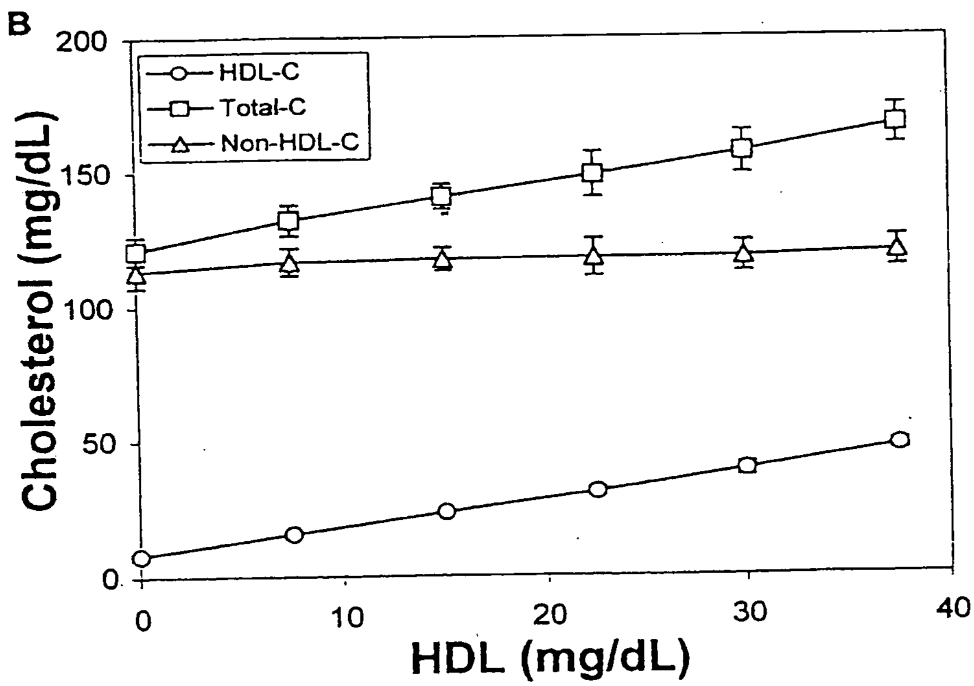
27. A kit of claim 22, wherein the complex-forming agent is a sulfated cyclodextrin.

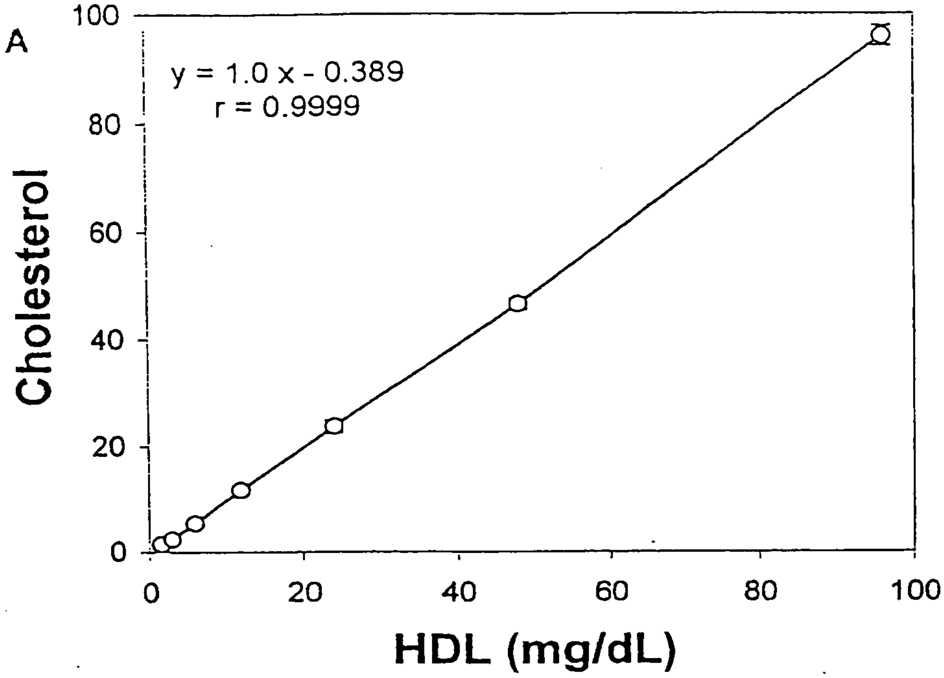
28. A kit of claim 22, further comprising one or more enzymes selected from the group consisting of lipase, glycerol kinase, glycerol phosphate dehydrogenase, glycerol phosphate oxidase, and peroxidase.

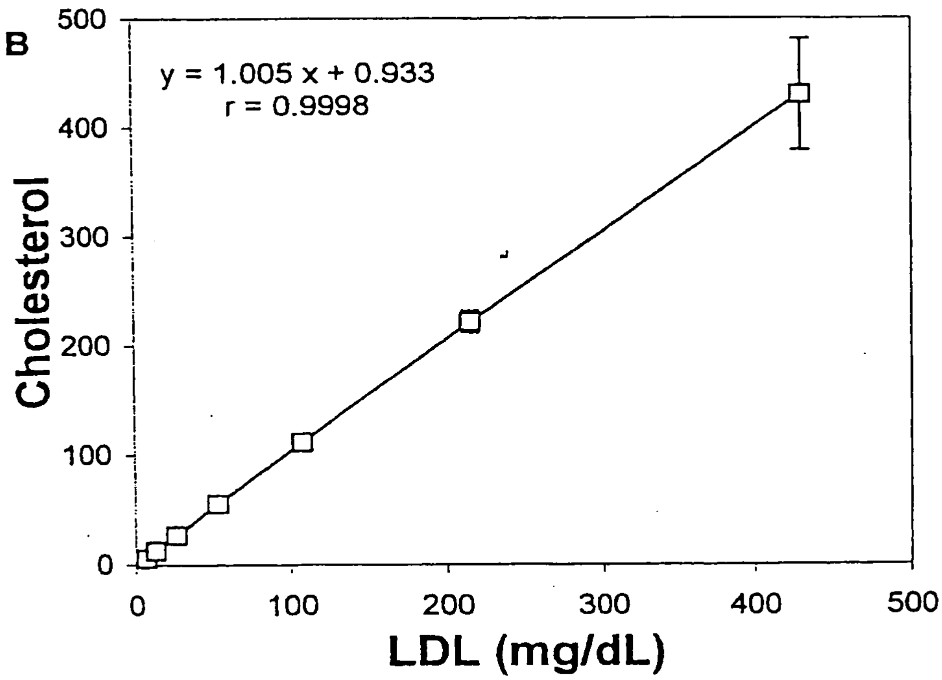
29. A kit of claim 22, further comprising one or more enzymes selected from the group consisting of pyruvate kinase, and lactate dehydrogenase.


Detergent disruption and
non-HDL-C measurement
HDL-C
measurement
non
HDL-C
Total-C
Ab blocking
of non-HDL
fraction
HDL-C
Absorbance
Time


A
HDL-C
Total-C
Non-HDL-C
Cholesterol (mg/dL)
0
50
100
150
200
0
20
40
60
80
100
120
LDL (mg/dL)


B
HDL-C
Total-C
Non-HDL-C
Cholesterol (mg/dL)
0
50
100
150
200
0
10
20
30
40
HDL (mg/dL)


A
y = 1.0 x - 0.389
r = 0.9999
Cholesterol
HDL (mg/dL)
0
20
40
60
80
100


B
y = 1.005 x + 0.933
r = 0.9998
Cholesterol
LDL (mg/dL)
0
100
200
300
400
500

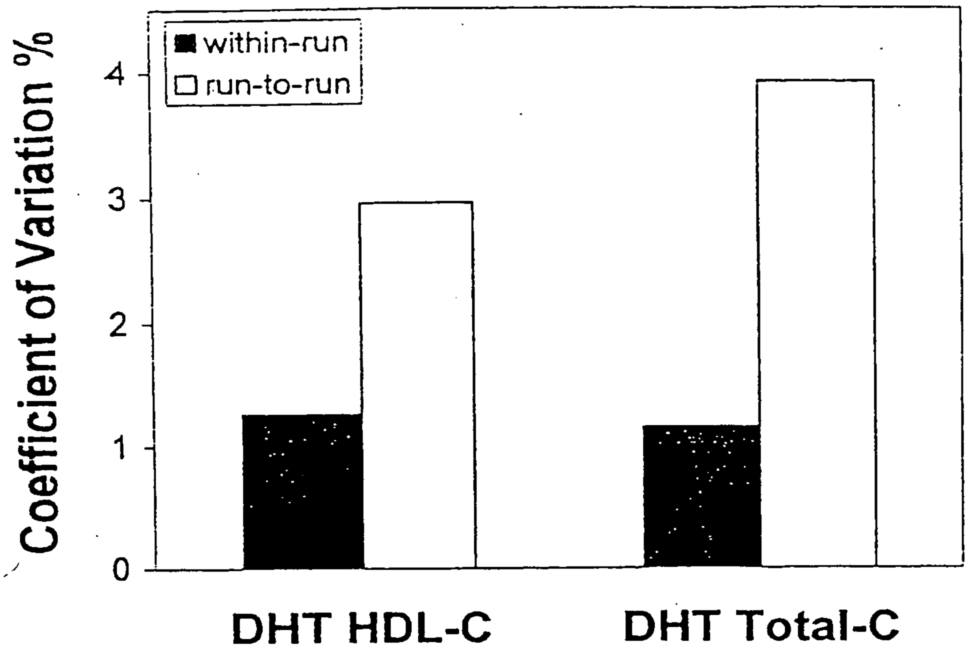

Coefficient of Variation %
within-run
run-to-run
0
1
2
3
4
DHT HDL-C
DHT Total-C

Fig. 6A

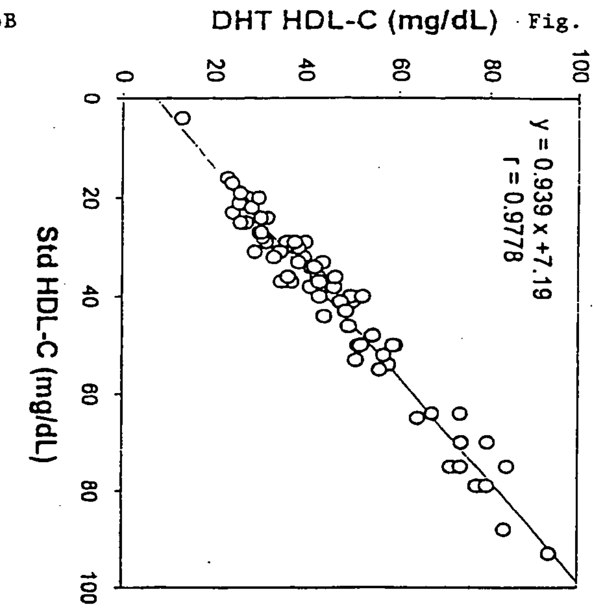


Fig. 6B

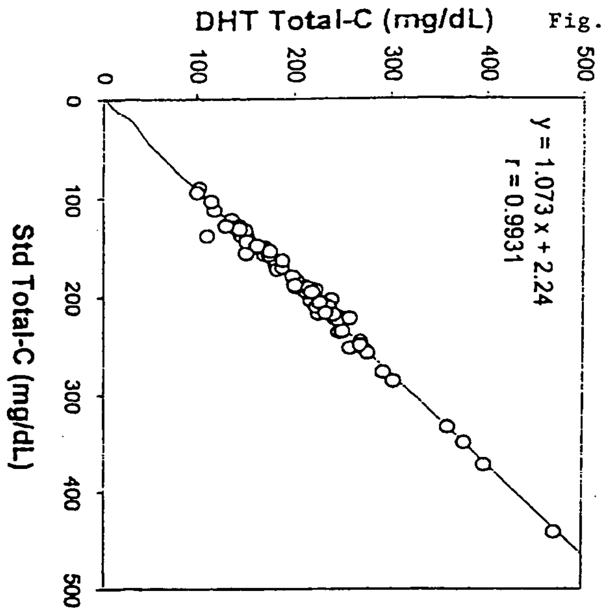


Fig. 6C

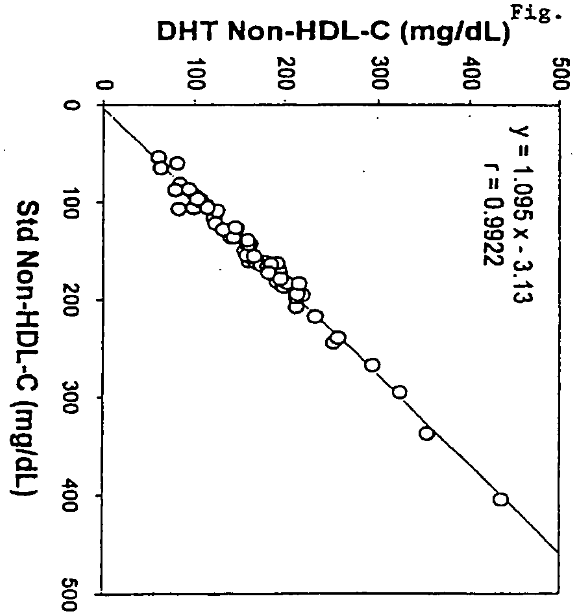


Fig. 6D

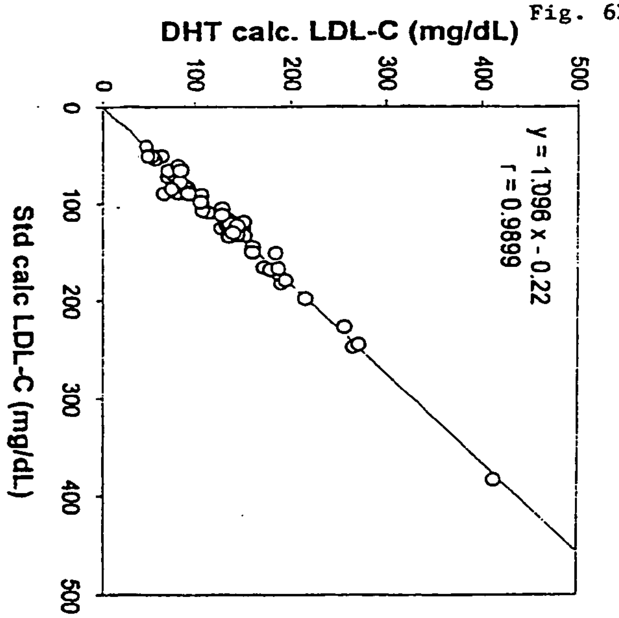


Fig. 2A

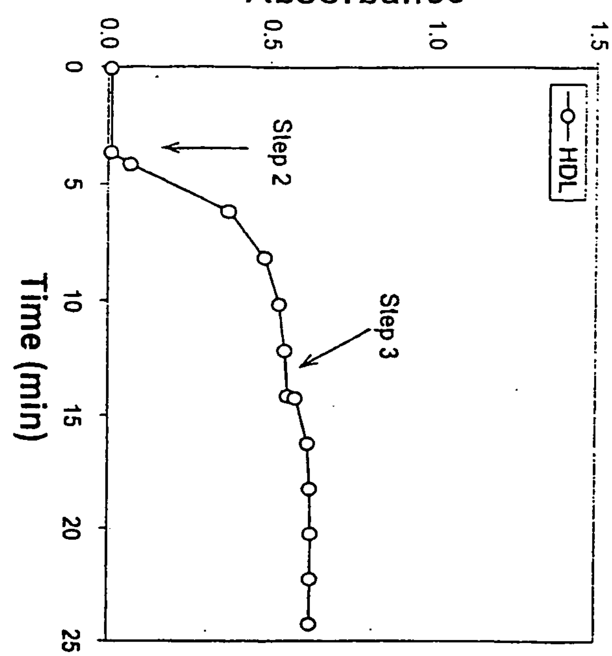


Fig. 2B

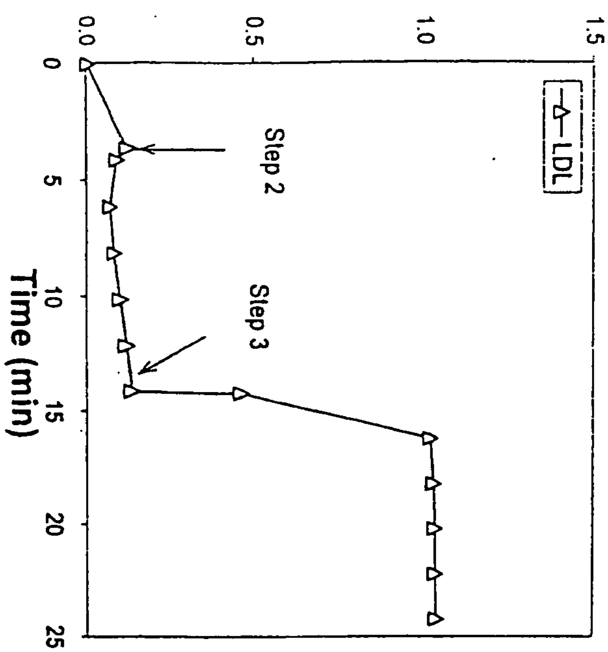


Fig. 2C

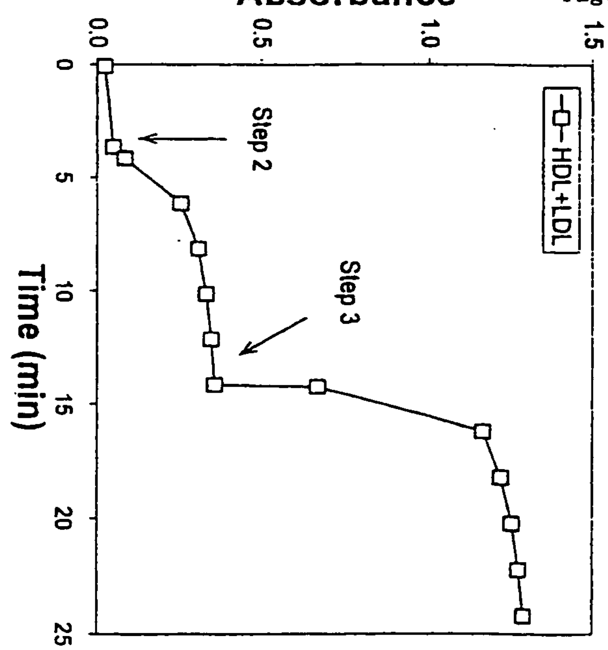


Fig. 2D